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ORIGINAL RESEARCH ARTICLE

Association between *PER3* length polymorphism and onco-hematological diseases and its influence on patients' functionality

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***Abstract*:** Circadian clock gene *PER3* and its length polymorphism may have a role in oncogenesis as clock genes act as key regulators of cell cycle and DNA repair pathways. The polymorphism may affect the condition of patients who show disrupted circadian rhythm due to tumor development. The aim was to assess the association between *PER3* polymorphism and onco-hematological diseases, and analyze whether this variant has an impact on patient's functionality. We conducted a case-control study on 125 patients with onco-hematological diseases and 310 control patients. *PER3* allelic variants were detected by using polymerase chain reaction. Sociodemographic data and information on patient's habits and functionality were obtained through questionnaire. Genotypes 4/5 + 5/5 showed an odd ratio (OR) = 1.39, with no statistical significance. However, those genotypes were associated with a two-fold increase in the risk of acute/chronic lymphoblastic/myeloblastic leukemia, taken all together. The occurrence of "changes in humor during last two months" was significantly associated with onco-hematological diseases. "Fatigue on awakening" and "self-reported snore" were associated with cases carrying the 4/5 or 5/5 genotypes. The results suggested that *PER3* polymorphism may have a role in the risk of leukemia, and might be a possible marker for individual differences in susceptibility to sleep disruption. This work provides insights for the identification of individuals at high risk of cancer, and those who are more susceptible to circadian disruption, which may decrease the physiological defenses against the tumor.

***Keywords*:** *PER3*; polymorphism; hematologic cancer; circadian rhythm; case-control study

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The circadian system regulates metabolism and energy homeostasis on a daily basis in order to maintain vital processes and prepare the organism to respond to predictable/daily environmental conditions[1]. Accordingly, most of the mammalian physiology is regulated at some points by the main circadian clock which is located at the hypothalamic suprachiasmatic nuclei (SCN). Circadian coordination is known to be

extremely important for healthy physical and mental function, as many diseases display disruption of circadian rhythms[2,3]. Several studies have reported circadian alterations in cancer patients and tumor-bearing animals[4,5]. Moreover, current data have suggested that this disruption could be more than a consequence of cancer development and it may act as a risk factor[6,7]. In 2007, the International Agency for Research on Cancer (IARC, World Health Organization) concluded that shiftwork that involves circadian disruption is probably carcinogenic to humans (Group 2A)[8]. Besides the core clock at the SCN, peri- pheral tissues also have circadian clocks which show internal desynchronization under pathological conditions[9].

At molecular level, the circadian clock involves transcriptional-translational negative feedback loops, rhythmic production and degradation of protein complexes that turn off their own production[10,11]. Some circadian genes involved in the loop also control the transcription of other genes, such as clock-controlled genes (*CCG*), which represent 2%–10% of the mammalian genome[12]. Although the majority of the *CCG* show tissue-specific expression patterns, a few sets of them are expressed in multiple organs and encode key regulators of the cell cycle, deoxyribonucleic acid (DNA) damage/repair pathways, and cell death[6,13].

Period (*PER*) genes are part of the core of the mechanism involved in the circadian clocks. Period circadian clock 3 (*PER3*) is a member of the *PER* family, and in humans it contains a variable number tandem repeat (VNTR) polymorphism, consisting of a 54-bp coding region repeated 4 or 5 times[14]. These repeats are of interest because it included numerous potential phosphorylation sites. Thus, they could affect post-translational modification and stability of the protein. Several studies hypothesized that *PER3* VNTR polymorphism may alter the susceptibility to cancer[15]. Moreover, variants in the circadian genes (*CRY2*, *PER1*, *NPAS2* and *CSNK1E*) have been associated with different types of cancer, including non-Hodgkin lymphoma, prostate and breast cancer[16-20]. Studies on the expression patterns of circadian genes (*PER3* and *CCG*) show that there are significant differences between tumor tissue and the normal one adjacent to the tumor[21-22]. All these data show a possible role of *PER3* and its allelic variants in oncogenesis, and its potential use as a susceptibility biomarker.

As mentioned above, many physiological processes are affected by the tumor development, resulting in disrupted circadian rhythms in cancer patients. On the other hand, the central clock regulates sleep, mood, food intake and attention[23]. The connection between cancer, clock and behavior is quite relevant, given that living with cancer is emotionally exhausting. In fact, cancer patients undergo sickness behavior, a cluster of symptoms that include lethargy, depression, fever, hyperalgesia and decreased social interaction, which might be the result of both the disease and the treatment[24]. *PER3* has been reported to play a role in modulating sleep homeostasis in humans[25]. Thus, the VNTR polymorphism may have an impact on the patient's performance while facing changes in the circadian rhythm.

According to the Atlas of Cancer Mortality published by the Ministry of Health[26], the onco-hematologic diseases (leukemia, lymphomas and multiple myeloma) were the cause of over 18,500 deaths in Argentina from 2007–2011. These disorders exhibit an incidence of almost 850,000 cases/year worldwide, as reported by the IARC in its previous GLOBOCAN 2012 report[27]. In recent years, only a few studies addressed the connection between blood cancer and circadian rhythm, since most of the work on the topic focused on breast, prostate and colon cancers.

The aim of this work is to study the association between the VNTR of *PER3* and onco-hematological diseases, and analyze whether this variant has an impact on the patient's functionality in terms of fatigue, sleep and humor, among other variables.

Materials and methods

A case-control study consisting of 125 patients with onco-hematological diseases and 310 control patients was conducted. All the participants were recruited between June 2013 and March 2015 at the Unit of Diagnosis, Treatment and Support for Hematological Diseases of Hospital Prof. Dr. Rodolfo Rossi (La Plata, Buenos Aires, Argentina).

The cases included patients diagnosed with acute lymphoblastic leukemia (ALL, $N = 10$), acute myeloblastic leukemia (AML, $N = 18$), chronic lymphoblastic leukemia (CLL, $N = 10$), chronic myeloblastic leukemia (CML, $N = 20$), multiple myeloma (MM, $N = 29$), Hodgkin lymphoma (HL, $N = 18$) and non-Hodgkin lymphoma (NHL, $N = 20$). The controls were patients frequently visiting the unit for routine checks of disorders unrelated to cancer, or preoperative blood analyses. All the participants resided in Argentina. Cases and controls with previous history of cancer or pathologies which are closely related to onco-hematological diseases were excluded from the study.

Patients participated in this study upon signing an informed consent. A questionnaire was used to obtain

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sociodemographic data and information about habits and functionality of patients (previous 2 months), such as changes in weight (± 5 kg), changes in appetite (either increase or decrease), changes in humor (worse or better), presence of physical/mental fatigue, difficulty in sleeping (especially when trying to fall asleep), fatigue on awakening, waking up several times at night, early morning awakening and difficulty to fall asleep again, snoring, and good sleep quality. All the surveys were conducted by the same person. Blood samples were collected and kept in tubes with ethylenediaminetetraacetic acid (EDTA). Then, the DNA was extracted from whole blood using salting out methods.

The detection of *PER3* allelic variants was performed by PCR, using the primers 5′-TGGTCCCAGCAGTGAGAGT-3′ forward and 5′-CCAGATGCTGCTCTACCTGAACC-3′ reverse. Reaction conditions were as follows, in a final volume of 15 µL: 1× buffer, 50 ng DNA, 0.25 mmol/L each primer, 200 µmol/L dNTPs, 1.5 mmol/L $MgCl_2$, 0.45 U *Taq* Platinum Polymerase (Life Technologies) and H_2O up to 15 µL. The PCR cycling consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 1 min, followed by annealing at 57°C for 1 min and elongation at 72°C for 1 min, with a final extension at 72°C for 5 min. The products (261 bp and 315 bp) were visualized in 2% (w/v) agarose gels, stained with GelRed (Biotium Inc.).

Odds ratio (OR) and confidence interval at 95% (CI95%) were calculated to assess the association between each variable studied and hematological disease. Chi-square (Chi^2) test was applied to obtain the statistical significance of the association. Analyses were performed with STATA 11.1[28] and Epidat 4.0[29]. Allele and genotype frequencies were calculated and tested for Hardy-Weinberg equilibrium using GenAlEx 6.5[30,31]. *P*-values ≤0.05 were considered statistically significant. The sample size of this study achieved 80% power to detect an OR = 2.00.

Ethics statement

All the procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and together with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The informed consent was obtained from all participants in this study and this study was approved by the ethics committee of the hospital.

Results

Study population

In this association study, a total of 125 cases were compared to 310 controls, and they were all patients from the Hospital Prof. Dr. Rodolfo Rossi. Demographic characteristics are listed in *Table 1*. The missing data for each variable were not included in the analysis or detailed in the tables. The maximum number of missing data in a variable was 18, representing 4.14% of the samples. The height and weight data were excluded due to >10% missing data. There was no significant difference in the mean age of cases and controls. Women showed lower risk of the disease compared to men (OR = 0.52, CI95% 0.34–0.82, $p = 0.003$). Higher levels of education (>12 years) were significantly related with an increased risk, compared to those who completed primary school or less (OR = 3.68, CI95% 1.82–7.40, $p < 0.001$ adjusted for age and sex). Marital status did not show relation with the disease.

PER3 polymorphism

Genotype and allele frequencies for the VNTR of *PER3* were in accordance with Hardy-Weinberg equilibrium (*Table 2*). Considering the 4/4 genotype as reference, genotype 4/5 and the homozygous 5/5 did not show association with hematological cancer (OR = 1.50, CI95% 0.95–2.35 and OR = 1.80, CI95% 0.84–3.95, respectively). The trending *p*-value was 0.039.

Genotypes 4/5 and 5/5 analyzed together showed an OR = 1.39, with no statistical significance ($p = 0.175$ adjusted for age, sex, educational level and city of residence). However, those genotypes were associated with a two-fold increase in the risk of ALL, CLL, AML and CML, taken all together (OR = 1.99, CI95% 1.06–3.74, $p = 0.032$ adjusted for age, sex, educational level, and city of residence). There was no significant association between the polymorphism and the other diseases (MM, HL and NHL data are not shown).

Functionality

With respect to functionality variables evaluated in the questionnaire, only “changes in humor” showed significant associations with the onco-hematological diseases under study (*Table 3*, OR and *p* values adjusted for age, sex, educational level and city of residence). Variables “better mood during the last two months” (OR = 4.28, CI95% 2.00–9.12, $p < 0.001$) and “worse mood” (OR = 2.00, CI95% 1.17–3.42, $p < 0.001$) were significantly associated with the disease.

Impact of *PER3* polymorphism on patient’s behavior

Table 4 shows the distribution of *PER3* genotypes in

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Table 1 Demographic characteristics of the population under study

	Cases $N = 125$	Controls $N = 310$	OR (CI95%)	p
	Mean (SD)	Mean (SD)		
Age (years)	48.5 (16.6)	51 (18.5)	–	0.507
	N (%)	N (%)		
Sex				
Male	74 (59.2)	134 (43.2)	Ref.	0.003
Female	51 (40.8)	176 (56.8)	0.52 (0.34–0.82)	
Education				
⩽7 years (completed primary school or less)	55 (44.0)	174 (56.3)	Ref.	
12 years (completed secondary school)	48 (38.4)	115 (37.2)	1.2 (0.73-1.95)	<0.001[a]
>12 years (completed college)	22 (17.6)	20 (6.5)	3.68 (1.82–7.40)	
Marital status				
Single	27 (21.6)	79 (25.5)	Ref.	
Partner/Married	86 (68.8)	162 (52.3)	1.7 (0.99–2.95)	0.055[a]
Divorced/Separated	5 (4.0)	27 (8.7)	0.64 (0.21–1.90)	0.420[a]
Widowed	7 (5.6)	42 (13.5)	0.75 (0.27–2.13)	0.595[a]

a: Adjusted for age and sex. OR (CI95%): odds ratio and confidence interval 95%. Ref: reference category. SD: standard deviation. $p < 0.05$ considered statistically significant.

Table 2 Association between *PER3* polymorphism and onco-hematological diseases. Genotype and allele frequencies for the VNTR are also shown

PER3 polymorphism	Cases $N = 120$ N (%)	Controls $N = 297$ N (%)	OR (CI95%)	p	Chi^2 & p trend
	N (%)	N (%)			
Allele frequencies					
4-rep	0.69	0.76	–	–	–
5-rep	0.31	0.24			
Genotype frequencies					
4/4	58 (48.3)	176 (59.3)	Ref.		
4/5	50 (41.7)	101 (34.0)	1.50 (0.95–2.35)	0.077	4.26
5/5	12 (10.0)	20 (6.7)	1.82 (0.84–3.95)	0.130	p trend = 0.039
4/4	58 (48.3)	176 (59.3)	Ref.	0.175[a]	–
4/5 + 5/5	62 (51.7)	121 (40.7)	1.39 (0.86–2.25)		
LMC/LLC/LMA/LLA ($N = 58$)					
4/4	23 (39.7)	176 (59.3)	Ref.	0.032[a]	–
4/5 + 5/5	35 (60.3)	121 (40.7)	1.99 (1.06–3.74)		

a: Adjusted for age, sex, educational level and city of residence. OR (CI95%): odds ratio and confidence interval 95%. Ref.: reference category. $p < 0.05$ considered statistically significant.

controls and cases for each behavior variables analyzed. Variables “fatigue on awakening” and “self-reported snore” were associated with cases carrying the 4/5 or 5/5 genotype ($p = 0.003$ and $p = 0.036$ respectively, adjusted for age and sex). The rest of the variables included in the questionnaire did not show statistically significant associations.

Discussion

Patients who go through an oncologic disease may undergo circadian disruption as a reaction of physiology to tumor presence or as the result of endocrine response to physical/emotional demands of the illness. Cancer patients take longer time to fall asleep, wake up more often, spend more time in bed and nap more frequently than healthy individuals[32,33]. On the other hand, genetic, environmental or behavioral factors may favor circadian disruption, predisposing patients to tumor development[34]. Several studies have demonstrated that long-term night shiftwork is a prognostic value for breast cancer[35,36].

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Table 3 Association between functionality variables and onco-hematological diseases

	Cases $N = 125$	Controls $N = 310$	OR (CI95%)	p
	N (%)	N (%)		
Weight				
No changes	50 (42.0)	140 (47.0)	Ref.	
Increase/decrease <5 kg	31 (26.0)	84 (28.2)	1.03 (0.61–1.74)	0.902
Increase/decrease ≥5 kg	38 (32.0)	74 (24.8)	1.43 (0.86–2.38)	0.161
Appetite				
No changes	72 (60.0)	188 (62.1)	Ref.	
Increase	20 (16.7)	58 (19.1)	0.9 (0.50–1.60)	0.721
Decrease	28 (23.3)	57 (18.8)	1.28 (0.75–2.17)	0.355
Humor				
No changes	50 (41.0)	163 (53.8)	Ref.	
Better mood	22 (18.0)	24 (7.9)	4.28 (2.00–9.12)	<0.001[a]
Worse mood	50 (41.0)	116 (38.3)	2.00 (1.17–3.42)	0.001[a]
Physical/mental fatigue				
No	60 (48.0)	128 (41.4)	Ref.	0.211
Yes	65 (52.0)	181 (58.6)	0.77 (0.49–1.19)	
Fatigue on awakening				
No	77 (62.1)	199 (64.6)	Ref.	0.623
Yes	47 (37.9)	109 (35.4)	1.11 (0.71–1.75)	
Difficulty sleeping				
No	67 (53.6)	175 (56.8)	Ref.	0.541
Yes	58 (46.4)	133 (43.2)	1.14 (0.73–1.77)	
Waking up several times at night				
No	44 (35.2)	122 (39.3)	Ref.	0.420
Yes	81 (64.8)	188 (60.7)	1.19 (0.76–1.89)	
Early morning awakening				
No	95 (99.0)	207 (96.7)	Ref.	0.252
Yes	1 (1.0)	7 (3.3)	0.31 (0.01–2.49)	
Snore				
No	44 (36.4)	100 (34.8)	Ref.	0.769
Yes	77 (63.6)	187 (65.2)	0.94 (0.59–1.50)	
Good sleep quality				
Yes	66 (54.1)	159 (51.3)	Ref.	0.599
No	56 (45.9)	151 (48.7)	0.89 (0.57–1.39)	

a: Adjusted for age, sex, educational level and city of residence. OR (CI95%): odds ratio and confidence interval 95%. Ref: reference category. $p < 0.05$ considered statistically significant.

In our study, behavior variables such as fatigue, difficulty in sleeping, waking up several times at night or changes in appetite or weight did not associate with hematological cancer. This is probably because the study was hospital-based and more than 75% of the control patients were taking medication at the time of the inter- view, e.g., for different cardiologic or gastric disorders (data not shown). Moreover, a survey including >1,700 individuals from Buenos Aires (Argentina), Sao Pablo (Brazil), and Mexico DF (Mexico) showed that 2/3 of interviewed people experienced some type of sleeping difficulty during the previous year, and more than 25% individuals were moderately/severely affected[37]. Among them, the most common sleep disturbances reported within the last 12 months were: waking in the middle of the night (65%), waking up tired (55%), difficulty in sleeping and restarting it after an interruption (50%), or waking up too early (35%)[37].

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Table 4 Distribution of *PER3* genotypes among cases and controls, and its association with functionality variables

	Cases		Controls		$p^{a,b}$
	4/4	4/5 + 5/5	4/4	4/5 + 5/5	
Weight					
No changes	22 (40.7)	27 (45.0)	69 (40.8)	62 (53.5)	
Increase/decrease <5 kg	12 (22.2)	18 (30.0)	53 (31.4)	28 (24.1)	0.318
Increase/decrease ≥5 kg	20 (37.1)	15 (25.0)	47 (27.8)	26 (22.4)	
Appetite					
No changes	32 (57.2)	35 (59.3)	101 (58.7)	80 (67.8)	
Increase	12 (21.4)	8 (13.6)	36 (20.9)	19 (16.1)	0.180
Decrease	12 (21.4)	16 (27.1)	35 (20.4)	19 (16.1)	
Humor					
No changes	21 (37.5)	26 (42.6)	87 (50.9)	69 (58.0)	
Better mood	12 (21.4)	10 (16.4)	16 (9.4)	8 (6.7)	0.561
Worse mood	23 (41.1)	25 (41.0)	68 (39.7)	42 (35.3)	
Physical/mental fatigue					
No	29 (50.0)	27 (43.6)	69 (39.4)	54 (44.6)	0.159
Yes	29 (50.0)	35 (56.4)	106 (60.6)	67 (55.4)	
Fatigue on awakening					
No	42 (72.4)	31 (50.8)	108 (61.7)	83 (69.2)	0.003
Yes	16 (27.6)	30 (49.2)	67 (38.3)	37 (30.8)	
Difficulty sleeping					
No	31 (53.4)	31 (50.0)	94 (53.7)	75 (62.5)	0.168
Yes	27 (46.6)	31 (50.0)	81 (46.3)	45 (37.5)	
Waking up several times at night					
No	21 (36.2)	21 (33.9)	66 (37.5)	53 (43.8)	0.335
Yes	37 (63.8)	41 (66.1)	110 (62.5)	68 (56.2)	
Early morning awakening					
No	42 (100.0)	48 (98.0)	118 (97.5)	82 (95.4)	0.990
Yes	0	1 (2.0)	3 (2.5)	4 (4.60)	
Snore					
No	25 (44.6)	19 (31.2)	47 (29.2)	43 (38.0)	0.036
Yes	31 (55.4)	42 (68.8)	114 (70.8)	70 (62.0)	
Good sleep quality					
Yes	32 (55.2)	31 (52.5)	87 (49.4)	67 (55.4)	0.320
No	26 (44.8)	28 (47.5)	89 (50.6)	54 (44.6)	

a: *p*-values for interaction between functionality variables and *PER3* genotype were estimated using a logistic regression model extended to include the interaction term. b: Adjusted for age and sex. $p < 0.05$ considered statistically significant.

In fact, sleeping difficulties affect a significant proportion of people living in urban areas who are experiencing social jet-lag and night light pollution[38,39]. Since these disturbances are even worse for chronic diseases, we did not observe significant differences between cases and controls in our study. However, "feeling positive or negative changes in humor" was significantly associated with onco-hematological diseases ($p < 0.001$ adjusted for age, sex, educational level and city of residence). Several factors in a cancer patient should be noted, such as history and family background, physical and psychological impact of the illness, psychic resources and medical care. Cancer patients often suffer from adjustment disorders and anxiety generated by the diagnosis, prognosis, the

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wait for results, family conflicts, fear of recurrence and death, abnormal metabolic states and drugs, such as corticosteroids[40]. Therefore, patient's mood will depend on the interaction between all these factors in a more complex scenario than any other chronic diseases. In fact, the depression in cancer patients is twice as likely compared to the patients hospitalized due to other medical problems[40].

In the present study, the VNTR polymorphism of *PER3* increased the risk of onco-hematological diseases by 39%. During the analysis of leukemia separately from the rest of the conditions, the genotypes 4/5 and 5/5 were observed to be associated with a statistically significant increased risk (OR = 1.99, CI95% 1.06–3.74, p = 0.030 adjusted for age, sex, educational level and city of residence). Individual studies showed no conclusive results for the above-mentioned polymorphism[19,20,41]. A meta-analysis carried out by Geng and colleagues[15] combining three retrospective studies (2,492 cancer patients and 2,749 controls) reported that individuals with 5 repetition alleles had 17% increased risk of cancer compared to individuals with the 4 repetition alleles. However, this association was not statistically significant. A study on an American population reported a significantly higher risk of breast cancer among premenopausal women with the 5 repetition alleles[16], but a larger replication with Chinese samples did not show significant association results[20]. So far, there are no studies which evaluate the possible association between the VNTR with any of the blood cancers.

When we analyzed the distribution of *PER3* genotypes among controls and cases, we found that cancer patients with the 4/5 or 5/5 genotypes had greater fatigue on awakening. Voinescu et al.[42] focused on *PER3* genotypes and applied a battery of questionnaires to a population with self-reported sleep problems, finding that homozygotes for the 5 alleles showed difficulties in getting up more frequently than those with the 4 alleles or heterozygotes. In another study, a group of 24 healthy volunteers were subjected to sleep deprivation. It was observed that during the morning hours of the second day of sleep deprivation which was approximately 2–6 h after the melatonin peak, the performance (working memory, attention and psychomotor performance) deteriorated significantly in 5/5 individuals, whereas the decline was lower in 4/4 ones[25,43]. Based on the available data described above, our results suggest that cancer patients with 4/5 or 5/5 genotype may suffer fatigue more intensely, since they combine circadian disruption from the pathology itself with an increased susceptibility to sleep deprivation due to *PER3* genotype. It is important to take into account that our results were obtained with a non-validated questionnaire and more accurate approaches could be achieved using validated tools.

Case-control studies, as other population analyses, should also consider the ethnic composition of the group under study as allele frequencies vary among different populations. For the same reason, caution should be exercised when extrapolating results from one population to another.

Conclusion

All these data show that the VNTR of *PER3* may have a role in the risk of leukemia, and it may be a significant marker for individual differences in sleep, vulnerability to sleep disruption and circadian phase misalignment. The investigations aimed at elucidating the molecular connection between circadian genes and carcinogenesis will be helpful in identifying individuals at a higher risk or more susceptible to circadian disruption. It is worth to note that circadian disruption may decrease the physiological defenses against the tumor.

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Conflict of interest

The authors declared no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

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